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Formation of formate and hydrogen, and flux of reducing equivalents and carbon in *Ruminococcus flavefaciens* FD-1

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Abstract

A pathway for conversion of the metabolic intermediate phosphoenolpyruvate (PEP) and the formation of acetate, succinate, formate, and H_2 in the anaerobic cellulolytic bacterium *Ruminococcus flavefaciens* FD-1 was constructed on the basis of enzyme activities detected in extracts of cells grown in cellulose- or cellobiose-limited continuous culture. PEP was converted to acetate and CO_2 (via pyruvate kinase, pyruvate dehydrogenase, and acetate kinase) or carboxylated to form succinate (via PEP carboxykinase, malate dehydrogenase, fumarase, and fumarate reductase). Lactate was not formed even during rapid growth (batch culture, μ = 0.35/h). H_2 was formed by a hydrogenase rather than by cleavage of formate, and ^{13}C-NMR and ^{14}C-exchange reaction data indicated that formate was produced by CO_2 reduction, not by a cleavage of pyruvate. The distribution of PEP into the acetate and succinate pathways was not affected by changing extracellular pH and growth rates within the normal growth range. However, increasing growth rate from 0.017/h to 0.244/h resulted in a shift toward formate production, presumably at the expense of H_2. This shift suggested that reducing equivalents could be balanced through formate or H_2 production without affecting the yields of the major carbon-containing fermentation endproducts.

Introduction

Ruminococcus flavefaciens is a Gram-positive, strictly anaerobic bacterium that is widely regarded as an important agent of cellulose digestion in the rumen (Stewart & Bryant 1988). The relative amounts of fermentation endproducts (acetate, succinate, formate, H_2, and under some conditions, lactate) appear to vary with strain and growth conditions. Pettipher & Latham (1979) have shown that cellobiose-limited continuous cultures of *R. flavefaciens* 67 produced increased amounts of formate and succinate, and decreased amounts of acetate and lactate, with increased growth rate. By contrast, strain FD-1 grown at different rates (0.017–0.101/h) in cellulose-limited chemostats did not produce lactate, and both the acetate and succinate yields displayed only slight fluctuations with changes in pH or growth rate (Shi & Weimer 1992).

Although limited enzymatic surveys regarding acetate and succinate formation have been reported in *R. flavefaciens* strain C grown in batch culture on glucose (Hopgood & Walker 1969) and strain C94 grown in batch culture on cellobiose (Joyner & Baldwin 1966; Joyner et al. 1977), interpretation of the flux of carbon and reducing equivalents is not possible in this species without knowing the pathway of formate production. The purpose of this study was to determine the pathway of formate and H_2 production in *R. flavefaciens*, and to explore the flux of reducing equivalents and carbon under different growth conditions.

Materials and methods

Culture and cell harvest. *R. flavefaciens* FD-1 was grown in cellulose-limited and cellobiose-limited continuous cultures at different combinations of pH (6.02–7.08) and dilution rates (D = 0.017–0.244/h) (Shi & Weimer, 1992). Fermentation endproducts were analyzed by HPLC (Weimer et al. 1991). H_2 could not be measured in the CO_2 sparged open culture system, but was instead estimated from redox balancing as described by Wood (1961); and calculated according to the assumption that mol H_2 = mol succinate + mol formate - 1.5 mol cells – 2 mol CO_2 consumed. CO_2 was calculated based on the assumption that one mol of CO_2 was produced per mol of acetate produced, and that one mol of CO_2 was consumed per mol succinate or formate produced; thus mol CO_2 produced = mol acetate - mol succinate – mol formate. Cells were harvested at steady state by collecting culture effluent on ice under CO_2, followed by centrifugation under CO_2 for 1 h at 15,000 × g and 4 °C. The cell pelllets were transferred into 5 ml serum bottles on ice in an anaerobic glove bag (Coy Instruments, Ann Arbor, Mich.) under 10% H_2/90% CO_2, and were stored at - 70 °C.

Cell extracts and protein assay. Cell extracts were prepared by passing a suspension of rapidly-thawed cell paste (1 g wet weight) in 1–2 ml TGD buffer (50 mM Tris-HCl pH 7.0, 13% [w/v] glycerol, 1 mM dithiothreitol [DTT]) [10] or TGDP buffer (50 mM triethanolamine-HCl pH 7.5, 13% [w/v] glycerol, 1 mM DTT, 2 mM pyruvate) through a French pressure cell at 1460 kg/cm^2 under N_2. The suspensions of broken cells were centrifuged at 37,500 × g and 4 °C for 1 h. Supernatant fluids (cell extracts) were used for enzyme assays immediately or were stored under N_2 at - 70 °C. Protein in cell extracts (2–16 mg/ml) was determined by the method of Bradford (1976) using commercial protein assay reagents (Bio-Rad, Richmond, Calif.) with lysozyme as a protein standard. Protein content of toluenized whole cells or of suspensions of cell debris was measured as described by Pavlostathis et al. (1988).

Enzyme assays. i) *Spectrophometric assay.* Reactions of pyruvate kinase (EC 2.7.1.40) (Collins & Thomas 1974); pyruvate dehydrogenase (EC 1.2.4.1) (Weimer 1984); pyruvate-formate lyase (EC 2.3.1.54) (Weimer 1984); hydrogenase (EC 1.18.3.1), (Weimer 1984); lactate dehydrogenase (EC 1.1.1.27) (Anonymous 1973); acetate kinase (EC 2.7.2.1) (Nakajima et al. 1978); PEP carboxykinase (EC 4.1.1.32) (Hopgood & Walker 1969); PEP carboxylase (EC 4.1.1.31) (Canovas & Kornberg 1969); PEP carboxytra sphosphorylase (EC 4.1.1.38) (Wood et al. 1969); malic enzyme (EC 1.1.1.40) (Hsu & Lardy 1969); pyruvate carboxylase (EC 4.1.1.31) (Seuber & Weicher 1969); malate dehydrogenase (EC 4.2.1.2) (Zeikus et al. 1977); and fumarate reductase (EC 1.18.3.1) (Thauer et al. 1977) were continuously monitored spectrophometrically. Reactions were started by addition of the specific substrate for each assay. The measured reaction rates were corrected for background rates determined in the absence of the initiating substrate. Absorbance readings were made in a Beckman DU-50 spectrophotometer using anaerobic cuvettes (Starna Cells, Atascadero, Calif.) having a 1 cm light path, 1 ml total reaction volume, 0.35 ml headspace, and a 39 °C incubation temperature. Formic hydrogenylase (EC 1.2.1.2) (Joyner & Baldwin 1966) was assayed by monitoring the production of hydrogen after 1 h incubation by cell extracts in the presence of reduced benzyl viologen, using a Shimadzu GC-8A gas chromatograph equipped with a Carbosieve SII column (60/80 mesh, 2.9 m × 0.32 cm diameter; Supelco, Bellefonte, Pa), a N_2 carrier gas, and a thermal conductivity detector.

ii) *Pyruvate exchange reactions.* Pyruvate-formate exchange assays were based on a modification of the method of Nakayama et al. (1971). The 1 ml reaction mixture contained: 20 mM potassium phosphate (pH 7.20), 50 mM sodium pyruvate, 1 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 0.7 mM DTT, 1 mM acetyl-CoA, 50 mM potassium-^{14}C-formate (8.8×10^3 dpm/μ mole), and cell extract (2.3 mg protein). A boiled cell extract was used as a negative control and a cell extract of *Escherichia coli* DH1 (3.6 mg crude protein) was used as a positive control (*E. coli* DH1 was generously provided by W.R. Kenealy, J. Whittier Biologics, Madison, Wisc.). All reagents except cell extract were added to Spinco polycarbonate centrifuge tubes (Beckman, Fullerton, Calif.) under a N_2 atmosphere. The assay was started by injecting cell extract, and incubations were performed in a water bath at 39 °C for 1 h. Trichloroacetic acid (0.3 ml of a 10% [w/v] solution) was injected to stop the reaction, and the protein precipitate was removed by centrifugation. To the supernatants 3 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl was added. The resulting hydrazones were collected by filtering the suspension through preweighed polycarbonate membranes (3 μm pore size, Nuclcopore, Pleasanton, Calif.), washing with 0.3 M HCl and final-

ly washing with water. The weights of the hydrazones were determined following overnight drying at 105 °C. The dried hydrazone and filter were placed into a liquid scintillation vial, and 200 μl of 1 M KOH was added. After the hydrazone was dissolved completely, 10 μl of the solution was analyzed in 10 ml Optifluor scintillation fluid (Packard Instruments, Meriden, Conn.) with a Packard 1600-TR liquid scintillation spectrometer; radioactivities were corrected with a quench curve constructed with ^{14}C-formate and various volumetric additions of nonradioactive pyruvate-dinitrophenylhydrazones in KOH.

$^{14}CO_2$-pyruvate exchanges were undertaken as described by Miller (1961) except that 10 ml Optifluor replaced Bray's solution as the scintillation cocktail. The initial reaction mixture contained 50 mM $NaH^{14}CO_3$ (1.1×10^4 dpm/μmole) and 11.5 mg extract protein/ml. Boiled cell extract was used as a negative control.

iii) *NMR measurements.* A 24 h-old, cellulose-grown culture of strain FD-1 0.1 ml) was inoculated into sealed 7 ml serum bottles that contained 5.7 ml of modified Dehority medium (Weimer et al. 1991) and either 10 mg ^{13}C-sodium formate (under a CO_2 gas phase) or 18 mg ^{13}C-sodium bicarbonate (under a N_2 gas phase). After 12 h incubation, 3 ml of culture was centrifuged anaerobically at 12,500 × g for 5 min. A portion (0.5 ml) of supernatant was transferred into 5 mm diamter NMR tubes that contained 50 μl deuterium oxide (as an internal reference) and 50 μl of 10 M NaOH (to prevent H-D exchange and to sharpen signals). Carbon-13 chemical shifts (relative to dioxane at 67.4 ppm) of putative substrates and products were determined in basic solution from NMR spectra recorded with a Bruker AMX360 spectrometer operating at 90.57 MHz using a 5 mm standard-geometry, four-nucleus probe. Important parameters were: spectral width, 21,700 Hz; 90° pulse width, 8.2 μs; acquisition time, 1.5 s for 64k data points; relaxation delay, 1s. Free-induction decays were apodized by exponential multiplication (line broadening = 1 Hz), zero-filled, and Fourier transformed to give spectra consisting of 64k real points.

Chemicals. All chemicals were reagent grade. ^{13}C-enriched sodium formate and sodium bicarbonate (99 atom % ^{13}C) were from Aldrich (Milwaukee, Wisc.). ^{14}C-sodium formate and sodium bicarbonate were from ICN Biomedical (Costa Mesa, Calif.). All other reagents were obtained from Sigma (St. Louis, Mo.).

Table 1. Rate of fermentation endproduct formation in chemostats and specific activity of measured enzymes in cell extracts

Growth condition	Ranges
Dilution rate (/h)	0.017–0.244
pH	6.02–7.08
Rate of fermentation endproduct formation	(nmol/min/mg protein)
Protein (mg/l) (estimate from cell mass)	190–230
Acetate	20–280
Succinate	17–127
Formate	8–359
Hydrogen	0–35
Specific activity of measured enzymes	(nmol/min/mg protein)
Pyruvate kinase	106–1300
Pyruvate dehydrogenase	500–1340
Acetate kinase	850–6900
Lactate dehydrogenase	< 3
PEP carboxykinase	30–170
PEP carboxytransphosphorylase	< 5
PEP carboxylase	< 9
Pyruvate carboxylase	3–19
Malic enzyme	< 8
Malate dehydrogenase	60–380
Fumarate dehydrogenase	60–380
Fumarate reductase	800–1500
Hydrogenase	60–130
Formic hydrogenlyase	NA[a]
CO_2 reductase	NA[a]
Pyruvate-formate lyase	NA[a]

[a] NA = Activity was not demonstrated by spectrophotometer, or gas chromatography.

Results

Acetate formation. Pyruvate kinase, pyruvate dehydrogenase and acetate kinase all displayed sufficient activities at high (presumably saturating) substrate concentrations to account for the rates of acetate production observed in the chemostat (Table 1). Pyruvate dehydrogenase activity was also confirmed with a $^{14}CO_2$-pyruvate exchange trial (113 nmol/min/mg protein, using cell extract prepared from a cellulose-limited continous culture at D = 0.06/h, pH = 6.60). Addition of the strong oxidant H_2O_2 at the end of the incubation resulted in removal of radiolabel, consistent with the enzymatic exchange of $^{14}CO_2$ into the relatively labile carboxyl group of pyruvate. These data indicate that acetate and CO_2 are formed from pyruvate cleavage. Although pyruvate could also be an

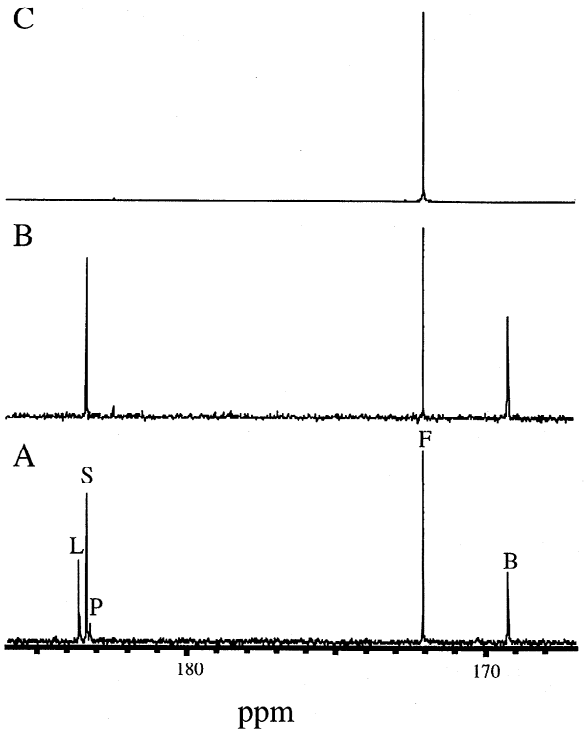

Figure 1. ^{13}C-NMR spectra of *R. flavefaciens* FD-1 batch cultures grown in the presence of ^{13}C-formate or ^{13}C-bicarbonate and treated with KOH. a. Carboxyl carbons in lactate (L), succinate (S), pyruvate (P), formate (F), and carbonate (B) standards. b. Cells grown in the presence of ^{13}C-bicarbonate, which was fixed into formate and succinate. c. In cells grown in the presence of ^{13}C-formate, exchanges of formate/pyruvate and formate/CO_2 did not occur.

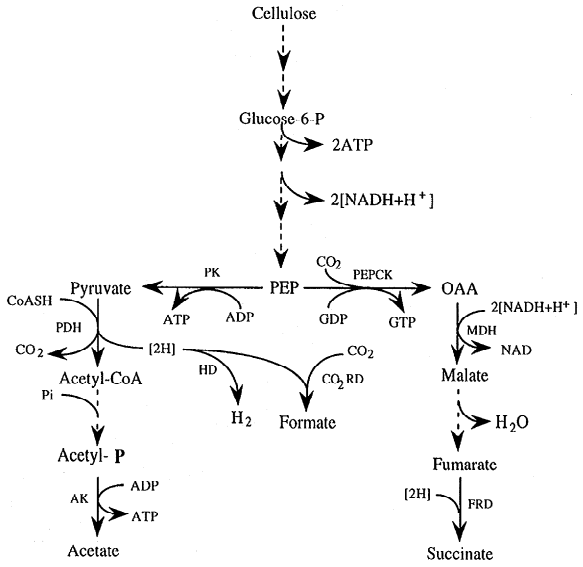

Figure 2. Pathway of fermentation endproducts in *R. flavefaciens* FD-1. AK = acetate kinase; CO_2RD = CO_2 reductase; FRD = fumarate reductase; HD = hydrogenase; MDH = malate dehydrogenase; OAA = oxaloacetic acid; PDH = pyruvate dehydrogenase; PEPCK = PEP carboxykinase; [2H] = unidentified reduced electron carrier. Solid lines indicate activities demonstrated in cell extracts, except for FRD, which was only observed in toluenized whole cells and cell debris. Dashed lines indicate activities not tested in this study, but reported previously (Hopgood & Walker 1969).

intermediate in the production of lactate, little or no lactate dehydrogenase was detected in enzymatic assays, a result consistent with the lack of measurable lactate observed in cultures under all experimental conditions.

Succinate formation. Succinate is normally produced via carboxylation reactions involving pyruvate or PEP (Mahler & Cordes 1971). Specific activities of PEP carboxykinase were sufficient to account for the rate of succinate formation observed in the chemostats (Table 1). Low activities of pyruvate carboxylase were detected in some cell extracts; activity was dependent on ATP, was inhibited by addition of avidin or oxalate, and was not activated by addition of acetyl-CoA. Slightly increased activities of pyruvate carboxylase were observed at high growth rates. Activities of PEP carboxytransphosphorylase, PEP carboxylase, and malix enzyme were < 10 nmol/min/mg protein.

Cell extracts prepared from either cellulose-limited continuous cultures (amended with sterile rumen fluid) or cellulose-grown batch cultures (amended with hemin) failed to show fumarate reductase activity, regardless of electron donor tested (NADH, NADPH, FADH, methylene blue, methyl viologen [MV], benzyl viologen [BV], or safranin). However, whole cell suspensions that were permeabilized with 1% toluene displayed very high rates (800–15000 nmol/min/mg protein) of oxidation of reduced BV upon addition of sodium fumarate. This BV-linked fumarate reductase activity was also observed in broken cell suspensiosn (10-fold diluted suspension from the French press, microcentrifuged at 12,500 × g for 2 sec, under CO_2). Using H_2 as electron donor without any artificial electron carrier in a toluenized, concentrated cell suspension (70 mg cell paste in 2 ml Tris-HCl buffer pH = 8.5), an increased H_2 consumption was observed when the fumarate was added (data not shown). These results suggest that fumarate reductase is present, and that it and/or its required cofactors are membrane-associated. Because the electrochemical gradient across the cell membrane was dissipated by toluene permeabilization or cell breakage, the possible coupling of fumarate reduction to the phosphorylation of ADP could not be demonstrated.

Table 2. Molar yields of fermentation endproducts at different growth rates

Substrate consumed in chemostat (/l)	μ (/h)	pH	Molar yields of fermentation endproducts[a]			
			Acetate	Succinate	Formate	H_2[b]
3.11 g cellulose[c]	0.020	6.80	0.820	0.590	0.297	0.590
3.25 g cellulose[c]	0.035	6.75	0.766	0.568	0.580	0.044
3.28 g cellulose[c]	0.059	6.83	0.846	0.685	0.684	- 0.019
0.92 g cellobiose	0.075	6.77	0.870	0.671	0.970	- 0.150
1.05 g cellobiose	0.244	6.75	1.106	0.506	1.431	- 0.123
3.97 g cellulose[c]	0.017	6.59	0.875	0.677	0.326	0.491
3.78 g cellulose[c]	0.044	6.56	0.793	0.573	0.335	0.377
3.23 g cellulose[c]	0.059	6.59	0.887	0.627	0.475	0.267
2.78 g cellulose[c]	0.078	6.60	0.706	0.400	0.423	0.224
2.67 g cellulose[c]	0.101	6.56	0.940	0.613	0.493	0.376

[a] mol fermentation endproducts per mol anhydroglucose equivalent of cellulose or cellobiose consumed.
[b] mol H_2 produced was not measured in the chemostat, but calculated according to the total redox balance: mol H_2 = (mol Succinate) + (mol Formate) - 1.5 (mol Cells) - 2 (mol CO_2 consumed). See Shi & Weimer (1992) for assumptions used in calculating mol cells.
[c] Data were from previous studies (Shi & Weimer 1992).

Formate formation. Two possible pathways were tested for production of formate: Cleavage of pyruvate via pyruvate-formate lyase or reduction of CO_2 via CO_2 reductase.

i) *Pyruvate cleavage pathway.* The specific radioactivities of ^{14}C-labeled pyruvate dinitrophenylhydrazones detected from ^{14}-Formate-pyruvate exchange were 49, 32, and 936 dpm/μmol for the *R. flavefaciens* FD-1 extract, boiled FD-1 extract (negative control) and *E. coli* DH1 extract (positive control), respectively. No pyruvate-formate lyase activity was detected spectrophotometrically in cell extracts.

ii) *CO_2 reduction pathway.* CO_2-formate exchange were examined by NMR with ^{13}C-labeled formate or CO_2. ^{13}C-NMR spectra from cultures grown on cellulose in the presence of ^{13}C-formate showed the formate peak at 172 ppm (Figure 1c). The lack of a bicarbonate signal at 169.2 ppm indicates that $^{13}CO_2$ was not formed from ^{13}C-formate. The labeling patterns of formate, succinate, and bicarbonate were obtained from ^{13}C-NMR spectra, using cells grown in ^{13}C–bicarbonate-supplemented media (Figure 1b). The data indicate that ^{13}C-bicarbonate was incorporated into the carboxyl groups of both formate and succinate. Results from the above measurements suggest that formate is not generated from the cleavage of pyruvate in strain FD-1, but is instead a product of CO_2 reduction. However, neither CO_2 reductase nor formate dehydrogenase could be demonstrated by spectrophotometric assay with any of several electron carriers tested (NADH, NADPH, FADH, MV or BV in the presence or absence of ferredoxin), with several enzyme sources (cell extracts or toluenized whole cells), or with several buffers in the pH range of 5.7–7.5.

Hydrogen formation. Hydrogenase activity was detected in all cell extracts assayed. Activities measured in the direction of H_2 oxidation (with BV as electron acceptor) were higher than the rate of H_2 production estimated in continuous cultures by redox balancing. Production of H_2 was detected by gas chromatography only in *E. coli* DH1 cell extract (positive control), but not in cell extracts of *R. flavefaciens* FD-1, after 1 h incubation in the presence of potassium formate and reduced BV. These data suggested that H_2 produced by strain FD-1 was formed from reduction of protons, not from cleavage of formate. This result differs from that of Joyner et al. (1977), who reported conversion of formate to H_2 (formic hydrogenylase activity) in cell extracts of cellobiose-cultured *R. flavefaciens* C94. In order to identify the *in vivo* electron carrier, attempts were made to couple H_2 oxidation to NAD^+ reduction via exogenously-added oxidized ferredoxin (from *Clostridium pasteurianum*) in two different buffers (CHES pH 9.0; Tris-HCl pH 7.4). No activity was detected from the same cell extract that showed a high hydrogenase activity with BV as electron acceptor.

Table 3. Ratios of fermentation endproducts under different growth rates and pH values

Run[a]	μ(/h)	pH	A/A+S[b]	s/A+S[b]	2S/2S+F+H[b]	F/2S+F+H[b]	H/2S+F+H[b]
1	0.019	7.05	0.60	0.40	0.55	0.10	0.35
2	0.020	6.80	0.58	0.42	0.59	0.15	0.26
3	0.017	6.59	0.56	0.44	0.62	0.15	0.23
4	0.020	6.26	0.56	0.44	0.62	0.21	0.18
5	0.019	6.02	0.63	0.37	0.50	0.20	0.30
6	0.020	6.06	0.56	0.44	0.60	0.20	0.20
7	0.027	6.38	0.66	0.34	0.46	0.32	0.22
8	0.029	6.03	0.60	0.40	0.59	0.27	0.14
9	0.035	6.75	0.57	0.43	0.65	0.33	0.03
10	0.044	6.56	0.58	0.42	0.62	0.18	0.20
11	0.048	6.14	0.59	0.41	0.59	0.29	0.12
12	0.059	7.08	0.52	0.48	0.75	0.33	- 0.08
13	0.059	6.83	0.55	0.45	0.67	0.34	- 0.01
14	0.059	6.59	0.59	0.41	0.63	0.24	0.13
15	0.061	6.60	0.62	0.38	0.55	0.28	0.17
16	0.060	6.40	0.54	0.46	0.70	0.39	- 0.09
17	0.059	6.16	0.55	0.46	0.68	0.31	0.01
18	0.076	6.46	0.64	0.36	0.53	0.25	0.22
19	0.078	6.60	0.64	0.36	0.55	0.29	0.16
20	0.101	6.56	0.61	0.40	0.59	0.24	0.18
21	0.075	6.77	0.56	0.44	0.62	0.45	- 0.07
22	0.244	6.75	0.69	0.31	0.44	0.62	- 0.05
Mean			0.59	0.41	0.60	0.28	0.13
s.d.[c]			0.04	0.04	0.07	0.11	0.13
Min			0.52	0.31	0.44	0.10	- 0.09
Max			0.69	0.48	0.75	0.62	0.35

Correlations coefficients (r) of growth rate (μ, in /h), pH, A/A+S, S/A+S, 2S/2S+S+F+H, F/2S+S+F, H/2S+S+F

Parameter	μ	pH	A/A+S	S/A+S	2S/2S+F+H	F/2S+F+H
pH	0.250					
A/A+S	0.483	- 0.105				
S/A+S	- 0.468	0.093	1.000			
2S/2S+F+H	- 0.318	0.181	- 0.941	0.946		
F/2S+F+H	0.785	0.134	0.219	- 0.218	- 0.091	
H/2S+F+H	- 0.490	- 0.219	0.360	- 0.362	- 0.504	- 0.814

[a] Runs 1–20, cellulose as substrate; runs 21–22, cellobiose as substrate.
[b] A = acetate, S = succinate, F = formate, H = hydrogen.
[c] s.d. = standard deviation; min = minimum value; max = maximum value.

Growth rate and fermentation endproduct shift. After the catabolic pathway (Figure 2) of this strain was established, the ratios of fermentation endproducts (acetate [A], succinate [S], formate [F], and H_2 [H] at different combinations of growth rate and extracellular pH were compared in two groups. The first group compared the partitioning of carbon into acetate (A/A+S) and succinate (S/A+S), because the carbon skeletons of both acetate and succinate were derived from PEP. The second group compared the partitioning of electrons into succinate (2S/2S+F+H), formate (F/2S+F+H), and hydrogen (H/2S+F+H), because formation of all three products required consumption of reducing equivalents (four for succinate, two for formate, and two for H_2 respectively). The data revealed that molar ratios of acetate and succinate were basically constant at 22 combinations of growth rates (0.017-0.244/h) and extracellular pH values (6.02–7.08) (Table 3). Of the

reducing equivalents not utilized in the synthesis of cell material, 60% were used for succinate production, regardless of extracellular pH and growth rate. The high negative correlation (r = - 0.814) between (F/2S+F+H) and (H/2S+F+H) and the positive correlation (r = + 0.785) between molar ratio of formate and growth rate suggested that a shift of metabolic pathway between the formations of formate and hydrogen occurred with changing growth rates (Table 3). In fermentations conducted at two similar pH values (pH 6.56–6.60; pr pH 6.75–6.83), a greatly increased production of formate and decreased production of H_2 (estimated from redox balancing) with increasing growth rates was observed (Table 2).

Discussion

Distribution of carbon flux in fermentations. Enzyme activities, ^{13}C-NMR data and ^{14}C-exchange experiments reveal that the carbon from the carbohydrate substrate was converted to acetate, succinate and CO_2 as sole catabolic products. Formate was formed from exogenous CO_2, probably via CO_2 reductase. Hydrogen was produced from reducing equivalents via hydrogenase. Data from enzyme assays (Table 1) suggest that the rates of acetate and succinate formation were not limited by the amounts of any enzyme in the pathway.

For succinate formation, activities of GDP-dependent PEP carboxykinase have been reported in *R. flavefaciens* strain C (Hopgood & Walker 1969), and malic enzyme has been suggested as the carboxylation enzyme in *R. flavefaciens* C94 (Joyner & Baldwin 1966). In our experiments, PEP carboxykinase alone among the five tested carboxylation enzymes showed activity sufficient to reflect the rate of succinate production in continuously-cultured cells of strain FD-1. PEP carboxylase, PEP carboxytransphosphorylase, and malic enzyme displayed very low, physiologically insignificant activities. Although pyruvate carboxylase measured in cell extracts was low, the specific activity did increase at higher growth rates. Operation of this enzyme would reduce the energetic efficiency of succinate production in rapidly growing cells, because the carboxylation of PEP-derived pyruvate would not provide a net gain of ATP, while direct carboxylation of PEP through PEP carboxykinase would provide a GTP.

Miller and Wolin (1973) observed that large amounts of ^{14}C-formate were produced by *R. albus* 7 when cultured in the presence of ^{14}C-bicarbonate, but very little $^{14}CO_2$ was produced when cultured in the presence of ^{14}C-formate. Moreover, they observed that this strain exchanged the carboxyl group of pyruvate with CO_2, but not with formate, and they conclude that formate arose from reduction of exogenous CO_2. By contrast, Joyner et al. (1977) suggested that pyruvate cleavage via pyruvate-formate lyase in *R. flavefaciens* C94 might provide formate for formic-hydrogen lyase, whose activity in this strain was high. Pettipher & Latham (1979) supported Joyner's suggestion by observing that addition of formate to cells from cellobiose-limited continuous cultures of *R. flavefaciens* 67 stimulated H_2 production. In our experiments with strain FD-1, no activities were shown in ^{14}C-formate-pyruvate exchange or in the spectrophotometric assay of pyruvate-formate lyase, suggesting that formate was not derived from pyruvate. No $^{13}CO_2$ was detected from ^{13}C-formate-CO_2 exchange reactions by NMR, and no hydrogen was observed in assays for formic-hydrogen lyase by gas chromatography, suggesting that formate was stable in the culture supernatant after it was produced. Although a direct demonstration of CO_2 reductase was not obtained from spectrophotometric assays, the data from $^{13}CO_2$-formate exchange in NMR assay showed clearly that formate can be formed from exogneous CO_2. Thus formate appears to be produced in this strain in the same way as in *R. albus* 7.

Enzymatic evidence for the capacity to reduce CO_2 to formate is usually provided by measuring the reverse reaction (formate dehydrogenase). Catabolic CO_2 reduction has been demonstrated in methanogenic and acetogenic bacteria, and normally requires various unique carrier molecules (Ljungdahl 1986; Mackie & Bryant 1994). Our failure to demonstrate CO_2 reductase activity could be due to the lack of the proper carrier molecules. Although ferredoxin is a commonly reported electron carrier for formate dehydrogenase in anaerobic bacteria (Ljungdahl 1986), no formate dehydrogenase was detected in extracts of strain FD-1 in the presence or absence of commercial clostridial ferredoxin.

Regulation of product formation. Dramatic shifts in carbon flux through catabolic pathways resulting from changes in growth rate or extracellular pH have been reported for several ruminal bacteria (e.g. *Streptococcus bovis* [Russell & Hino 1985] and *Selenomonas ruminantium* HD4 [Melville et al. 1988a, b]). These shifts are thought to allow bacteria to maintain an internal redox balance while adjusting the demands

for rapid ATP production at high growth rates with the demands of maximum catabolic efficiency (ATP yield) at low growth rates. By contrast, our studies with *R. flavefaciens* FD-1 have shown that yields of two carbohydrate-derived endproducts (acetate and succinate) are basically constant within the organism's physiological range of pH and growth rate. If ATP was generated during reduction of fumarate to succinate (as occurs in a number of succinate-producing anaerobes [Zeikus et al. 1977]), equivalent amounts of energy would be conserved from conversion of PEP to either acetate or succinate, and variable partitioning of carbon into the two pathways would have no effect on energy yield. Thus regulation of carbon flux would be unnecessary from the standpoint of maximizing ATP yield.

Because acetate production from PEP produces one pair of reducing equivalents, while succinate production consumes two pairs of reducing equivalents, regulation of carbon flux through the PEP branch point could potentially serve as an effective means of controlling the disposal of reducing equivalents necessary to maintain an internal redox balance. However, *R. flavefaciens* FD-1 appears to have adopted a simpler strategy involving formate and H_2 production. Results from the calculation of correlations (Table 2) and from molar yields of formate at similar pH conditions but varied growth rates (Table 3) suggest that regulation of the disposal of reducing equivalents in this strain mainly occurs through the production of formate and H_2, and the major flux of carbohydrate-derived carbon was not affected by this regulation.

Regulation of the disposal of excess reducing equivalents via formate in this strain is more advantageous for the bacterium than disposal via lactate, because carbon-containing fermentation intermediates remain available for further catabolism via energy-yielding reactions. Disposal of electrons via formate would also be thermodynamically favored by the high concentration of CO_2 found in the rumen and used in our *in vitro* experiments. However, a more complete understanding of the factors controlling formate and H_2 production will require determination of the kinetic parameters of the relevant enzymes, identification of their *in vivo* electron carriers, and measurement of intracellular and extracellular concentrations of H_2 and formate.

Ecological implications. In the rumen, methanogenic bacteria effectively utilize H_2 as electron donor in the reduction of CO_2 to methane. The continual removal of H_2 enhances the thermodynamics of H_2 production by fermentative microbes, resulting in increased flux of reducing equivalents through H_2. These 'interspecies H_2 transfer' reactions have been documented in numerous studies with both mixed ruminal microbes and defined bacterial cocultures (reviewed by Wolin 1977 and by Wolin & Miller 1983). Indeed, *Methanobrevibacter ruminantium* has been shown to dramatically alter the cellulose fermentation of *R. flavefaciens* C94, resulting in production of H_2 at the expense of succinate. The standard reduction potential for CO_2 to formate ($E^{\circ\prime}$ = - 432 mV) is similar to that for protons to H_2 ($E^{\circ\prime}$ = - 414 mV) (Thauer et al. 1977), and in the rumen both H_2 and formate are maintained in only trace quantities due to rapid consumption by methanogens (Hungate et al. 1970; Smolenski & Robinson 1988). Thus the partitioning of electrons into CO_2 reduction to formate or proton reduction may occur simultaneously and to similar extents, ultimately yielding the same product. Succinate yield, while decreased in the presence of methanogens, would probably be similar regardless of whether the flux of reducing equivalents was via H_2 or formate.

Industrial implications. Regardless of the effect of methanogens on succinate yield in *R. flavefaciens* in the rumen, the disposal of electrons as H_2 or formate may be of commercial significance. Cocultures of *R. flavefaciens* and *Clostridium kluyveri* have been shown to convert mixtures of cellulose and ethanol to butyric and caproic acids, potential liquid fuel precursors (Kenealy et al. 1995). In this coculture, *R. flavefaciens* ferments cellulose to succinate and acetate, which are converted by *C. kluyveri* in the presence of ethanol to butyrate and caproate. The variable disposal of reducing equivalents by reduction of protons to H_2, or to CO_2 to formate, allows *R. flavefaciens* to maintain consistent and high yield of succinate, the preferred organic acid substrate for *C. kluyveri*.

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